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Genetics

Identification of amber and ochre mutants of the human virus Ad2$^+$ND1

(yeast/tRNA/suppression/cell-free protein synthesis/host-range mutants)

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ABSTRACT **Although human adenoviruses grow poorly in monkey cells, this defect can be overcome either by coinfection of cells with simian virus 40 (SV40) or by insertion of the relevant portion of the SV40 genome into the adenovirus genome to form an adenovirus–SV40 hybrid virus. The nondefective adenovirus-2–SV40 hybrid virus, Ad2$^+$ND1, contains an insertion of 17% of the SV40 genome, which codes for at least part of a 30,000 dalton protein. A set of Ad2$^+$ND1 host-range mutants that have lost the ability to grow in monkey cells and behave as point mutants fail to synthesize the 30,000 dalton ND1 protein. Translation *in vitro* of SV40-specific mRNA from mutant-infected cells produces unique short polypeptides instead of the 30,000 dalton protein.**

Here we show that this set of host-range mutants includes both ochre and amber nonsense mutations. When the SV40-specific mRNA from the host-range mutants is translated *in vitro* to produce the polypeptide fragments, yeast suppressor tRNA can partially restore synthesis of wild-type-size 30,000 dalton protein. By this assay, one mutant is ochre and two are amber.

Protein synthesis is terminated by UAG (amber), UAA (ochre), and UGA codons in messenger RNA. When a nonsense mutation generates one of these triplets in phase within the coding region of a message, premature termination of polypeptide synthesis occurs at the site of the chain-terminating codon. In both bacteria and fungi, nonsense mutants have been extremely valuable genetic tools and have been used to identify the protein products of many specific genes. Because the truncated polypeptides synthesized by nonsense mutants are, in general, inactive, one must obtain permissive conditions in which the activity of the corresponding wild-type protein is not required or the effects of the mutation are suppressed. Suppressor mutations can be found that allow the cell's protein synthetic machinery to sometimes insert an amino acid at the position of the nonsense codon, restoring at least partial activity. Therefore, normally lethal mutations can be carried in a suppressing background while the phenotypic properties of the mutation can be studied in a nonsuppressing background (for review, see ref. 1).

Among eukaryotes, fungi provide the only clear-cut examples of organisms that contain nonsense mutations and suppressors. The elegant work of Sherman and his colleagues has shown that yeast suppressors cause the insertion of tyrosine or serine at the position of defined nonsense (UAG or UAA) mutations in the gene specifying iso-1-cytochrome *c* (2–4). It has recently been shown (5, 6) that the mutations genetically defined as nonsense suppressors in yeast cells are indeed mutations that result in altered tRNAs that allow reading of nonsense codons. The yeast suppressor tRNAs work in mammalian cell-free systems to suppress known bacteriophage amber and ochre codons.

So far, neither a nonsense mutant nor a corresponding suppressor has been described for higher eukaryotes. Suppressor mutations in mammalian cells would be extremely useful for genetic analyses. Similarly, nonsense mutants would be valuable tools in the identification of gene products by giving one the ability to recognize protein fragments. Here we show that three host-range mutants of the adenovirus 2 (Ad2)–simian virus 40 (SV40) hybrid virus, Ad2$^+$ND1, are nonsense mutants because termination of protein synthesis at the mutant site can be suppressed *in vitro* in a mammalian cell-free translation system using amber and/or ochre suppressor tRNA from yeast cells.

Human adenoviruses do not grow efficiently in monkey cells unless they are provided with a helping function by SV40 (7). Lewis *et al.* (8) isolated a series of nondefective Ad2–SV40 hybrid viruses that grow equally well in monkey cells and in the normal permissive human cell lines. One of these, Ad2$^+$ND1, contains an insertion of 17% of the SV40 genome in place of a 5.5% segment of adenovirus DNA (Fig. 1) (9–11). With the hope of finding specific mutations within the integrated SV40 region that apparently provides the helper function, Grodzicker *et al.* (21, 22) isolated seven nitrous acid-induced host-range mutants of Ad2$^+$ND1 that still grew normally on human cells but had lost the ability to grow efficiently on monkey cells. These mutants retain the SV40 sequences present in Ad2$^+$ND1 and can still be complemented for growth in monkey cells by SV40, although they fail to complement one another. Examination of the proteins made in permissive human infected cells shows that Ad2$^+$ND1 directs the synthesis of a protein of molecular weight (M_r) 30,000 not made by Ad2 (21, 23) and that three host-range mutants fail to make the M_r 30,000 ND1 protein (22). In at least two of the three cases, revertants can be easily isolated and they produce the wild-type protein, suggesting that the mutations are simple point mutations.

The M_r 30,000 ND1 protein is encoded, at least in part, by the SV40 insertion in the hybrid virus. Thus, the messenger species for this protein can be translated *in vitro* from Ad2$^+$ND1 mRNA that has been selected by hybridization to SV40 DNA (20). This message is transcribed from sequences about 1000 base pairs to the left of the SV40 insertion and going into SV40 sequences corresponding to the 3′-terminal region of early SV40 mRNA (12, 19) (see Fig. 1). Size considerations suggest that the NH_2-terminal end of the M_r 30,000 protein probably is translated from adenovirus sequences just to the left of the insertion, so that the protein is likely a hybrid one containing partly adenovirus-encoded and partly SV40-encoded sequences. Translation of mRNA from the host-range mutants shows that those viruses that do not make the M_r 30,000 product instead direct the synthesis of defined protein fragments (22). By adding tRNA derived from yeast nonsense suppressors to the cell-free system, we can ask whether these protein frag-

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Abbreviations: Ad2, adenovirus 2; SV40, simian virus 40; M_r, molecular weight; $NaDodSO_4$, sodium dodecyl sulfate.

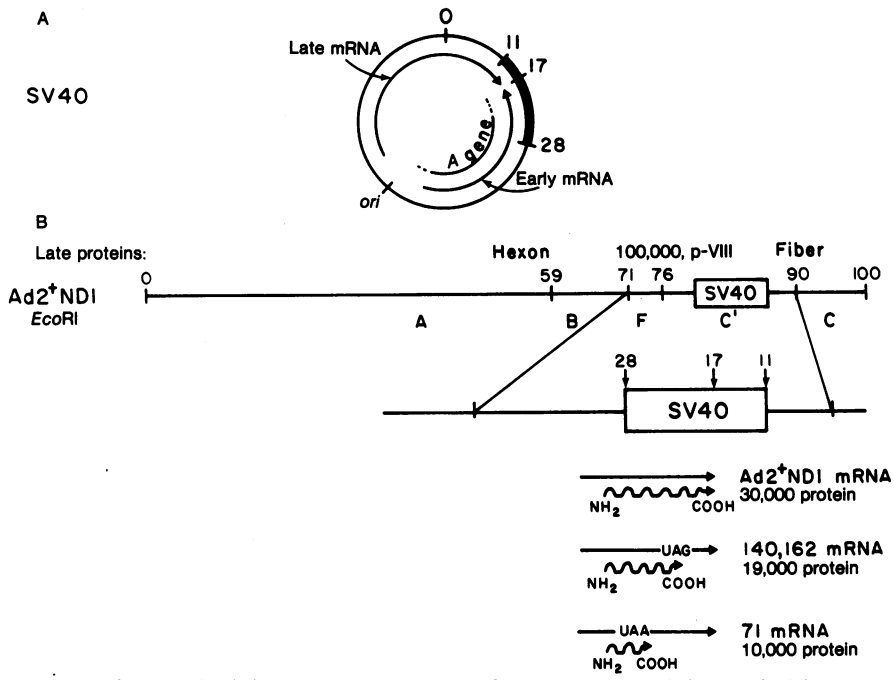

FIG. 1. Map of the genomes of SV40 and Ad2+ ND1. (*A*) The region of SV40 present in Ad2+ND1 (dark bar on outer circle) (10–12) as well as the origin of DNA replication (*ori*) and the regions of early and late cytoplasmic transcripts (13–15). (*B*) Map of the Ad2+ND1 genome shows the position of *Eco*RI cleavage sites (16, 17). The numbers above the line show the positions of cleavage sites; the letters below the line show the names of the DNA fragments produced. The positions of structural genes for several late adenovirus proteins, in relation to the Ad2 *Eco*RI fragments, are shown above the Ad2+ND1 map (18) (C. Anderson, unpublished data). The SV40 insertion is shown as a box in the Ad2+ND1 genome, and positions along the SV40 genome (positions 28, 17, and 11) are shown in the expansion below the map. The positions of Ad2+ND1 cytoplasmic RNA sequences are shown below the SV40 insertion (12, 19) (A. Dunn and J. Hassell, unpublished data). The ND1 M_r 30,000 protein (⁓) can be translated from Ad2+ND1 mRNA selected by hybridization to SV40 DNA (20) although the exact positions of the coding sequences are not shown. The short proteins produced upon translation of several SV40-selected Ad2+ND1 hybrid (mutants 140, 162, 71) mRNAs (21) are shown at the bottom along with the approximate positions of the chain-terminator mutants present in their DNAs (see *Results*).

ments result from termination at mutant ochre or amber sites.

MATERIALS AND METHODS

Cells and viruses were grown and assayed as described (22). Cytoplasmic RNA was prepared from suspension cultures of KB or HeLa cells at late times (22–30 hr) after infection with Ad2+ND1 or its host-range mutants as described (24). The SV40-specific mRNA was partially purified by hybridization to SV40 as described by Lewis *et al.* (18); this resulted in a 10- to 20-fold enrichment for mRNAs containing SV40 sequences compared to most other Ad2 mRNAs. As noted earlier (20), the Ad2 mRNAs for the M_r 100,000, M_r 38,000, and p-VIII proteins, which map immediately to the left of the SV40 insertion in Ad2+ND1, are also somewhat enriched by selection for mRNAs containing SV40 sequences.

The micrococcal nuclease-treated reticulocyte lysate system was prepared and used as described by Pelham and Jackson (25). A 0.4-ml frozen aliquot of reticulocyte lysate was diluted 1:1.25 by the following additions to give: 0.2 mM hemin (Eastman), creatine kinase (Sigma), 40 μg/ml; 10 mM creatine phosphate (Calbiochem); 100 mM KCl; 0.5 mM $MgCl_2$; 0.25 mM each of 19 amino acids (lacking methionine), excluding contributions from the lysate itself. Then the mixture was made 1 mM in $CaCl_2$ and 10 μg/ml in micrococcal nuclease (P-L Biochemicals) and incubated for 14 min at 20°. The nuclease action was terminated by addition of ethylene glycol-bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid to 2 mM. The mixture was added to 0.5 mCi of lyophilized [^{35}S]methionine (Amersham) (400–600 Ci/mmol), and rabbit liver tRNA (Sigma) was added to 37 μg/ml. Aliquots (12 μl) were then added to tubes containing 3 μl of total mRNA [final concentrations: 300 μg/ml for QB RNA, 500 μg/ml for total cytoplasmic RNA for adenovirus RNA; for hybridized RNA, see Anderson *et al.* (20)], yeast tRNA (as indicated), and water. After incubation for 60 min at 30°, a 2-μl aliquot was removed, treated with KOH and trichloroacetic acid, filtered, and assayed for radioactivity as described (25). Sodium dodecyl sulfate ($NaDodSO_4$)-containing sample buffer for polyacrylamide gel electrophoresis was added to the remainder at a ratio of 4/1 (vol/vol). After heating to 95° for 3 min, 5 μl was applied to 17.5% polyacrylamide gels made and run as described (26). After the gels were stained with Coomassie blue, destained, and dried *in vacuo*, the radioactivity was detected by autoradiography or fluorography (27).

Yeast strain DBA317 is a recessive lethal amber-suppressing strain isolated by Brandriss *et al.* (28) originally from the non-suppressing strain DBD195. It carries a suppressor, RL1, that maps near the mating type locus on chromosome 3 and inserts serine in response to an amber codon (28, 29). The suppressing activity resides in the tRNA fraction (5), and preliminary evidence suggests that a serine UCG-decoding species contains an altered anticodon corresponding to UAG (P. W. Piper, personal communication). Yeast strain H159, obtained from J. Hicks and G. Fink, carries the ochre suppressor *sup*61 first found by Hawthorne and Leupold (30), which maps in the same region of chromosome 3 and the RL1 suppressor and is probably allelic with it. It thus presumably inserts serine but in response to an ochre codon. Total tRNA was extracted and purified (5). The tRNA from both these strains was fractionated to enrich for the two suppressing species (unpublished data) by sequential chromatography on BD-cellulose and Sepharose 4B (31). The suppressing activity together with serine-chargeable species were strongly retarded by both columns. The final pooled fractions were about 50% pure with respect to serine-charging activity and, when analyzed on acrylamide gels, gave two principal components: one that migrated with the mobility of

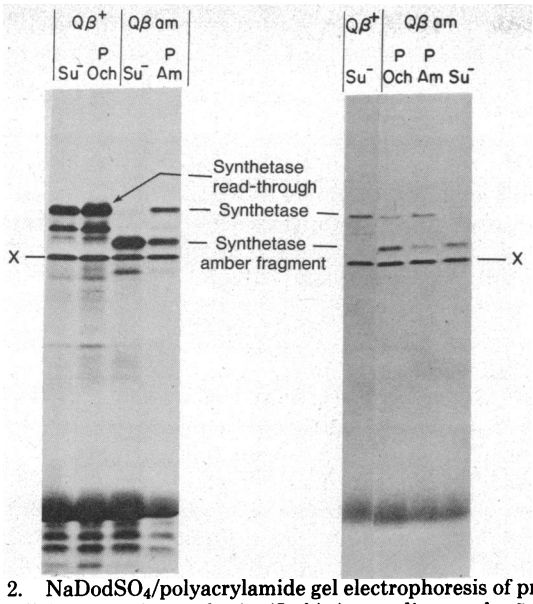

FIG. 2. NaDodSO$_4$/polyacrylamide gel electrophoresis of products of cell-free protein synthesis. (*Left*) Autoradiogram by 5-day exposure. (*Right*) Autoradiogram by 2-day exposure. The mRNAs are wild-type Qβ (Qβ$^+$) and the synthetase amber mutant of Qβ (Qβ am). Crude tRNA (*Su*$^-$, Och) or semipurified species (P Och, P Am) are from strains described in *Materials and Methods*. The synthetase read-through protein is only slightly (M_r 2700) larger than the normal Qβ synthetase (M_r 65,000) and is not well resolved from it in this gel system (5).

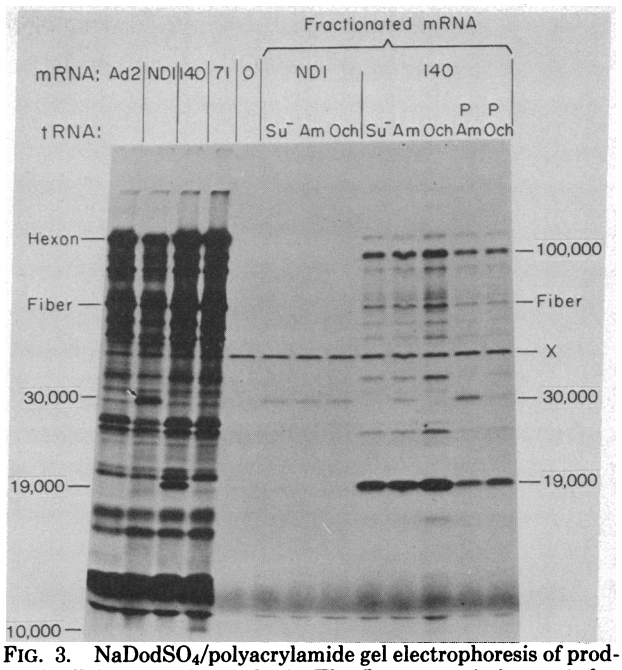

FIG. 3. NaDodSO$_4$/polyacrylamide gel electrophoresis of products of cell-free protein synthesis. The fluorogram is from a 5-day exposure of a dried 17.5% polyacrylamide gel. Samples are [^{35}S]methionine-labeled products from the nuclease-treated reticulocyte lysate cell-free extracts. Total cytoplasmic RNA was used as mRNA for the first four lanes (Ad2, ND1, 140, 71). Lane 0, incubation without added mRNA. The fractionated mRNAs ND1 and 140 are from hybridization of the total cytoplasmic RNA to SV40 DNA. The tRNAs added are crude (*Su*$^-$, *Am*, and *Och*) and partially purified (P Am, P Och) species as described in the *text*. Radioactivity in band X is observed even if protein synthesis is inhibited with cycloheximide.

serine tRNA and a faster-moving tRNA of unknown identity. A similar purification of serine tRNA from DBD195, the nonsuppressing parent of DBA317, was carried out. These semipure fractions were used in the experiments described below at final concentrations of 20–40 μg/ml compared to 80–150 μg/ml for the crude tRNAs.

RESULTS

***In Vitro* Assay for Nonsense Mutants.** Yeast ochre and amber tRNAs will give suppression in the micrococcal nuclease-treated reticulocyte lysate system described by Pelham and Jackson (25). When RNA from bacteriophage Qβ was translated, the M_r 65,000 synthetase protein could be detected by electrophoresis of the products on NaDodSO$_4$/polyacrylamide gels (Fig. 2). Note that the component marked "X" appeared in all samples, even those without added mRNA (see Fig. 3, lane 0) and in the absence of protein synthesis (data not shown). Presumably this was due to addition of [^{35}S]methionine to some component of the system. Translation of RNA from a Qβ mutant, *am*1, carrying an amber mutation in the synthetase gene gave a M_r 55,000 fragment (5) (K. Horiuchi, personal communication) (Fig. 2). Yeast tRNA from an amber-suppressing strain partially restored synthesis of the M_r 65,000 synthetase from the amber mutant. Ochre tRNA produced a read-thrugh protein from the wild-type synthetase, because UAA is apparently the normal terminator. However, addition of crude yeast tRNA, whether from a suppressing or a nonsuppressing strain, inhibited total protein synthesis. This inhibition could be partially overcome by fractionation of the tRNA species to enrich for the suppressing component. With the fractionated amber tRNA, we observed up to 80% suppression of the Qβ amber mutation, and with the fractionated ochre we observed up to 25% ochre suppression, as measured by read-through synthesis (data not shown). [In studies with the cell-free system of Schreier and Staehelin (24, 32, 33), the ochre tRNA gave >60% suppression (unpublished data).] Both the crude and the purified amber tRNA were specific for amber suppression and showed no ochre suppression. The purified ochre tRNA, on the other hand, had amber-suppressing activity (Fig. 2) that could not, however, be detected with the crude ochre tRNA. Thus, as the ochre species was purified, it gained the ability to suppress amber mutations. This activity was inconsistent with the strict specificity of this suppressor *in vivo* but resembled rather the activity of bacterial ochre suppressors that suppress both ochre and amber termination (34). The new ability to suppress amber could be due to loss of some modification of the tRNA during the course of purification or could be merely due to the fact that more of the purified species could be put into the reaction mixture, which revealed a low-efficiency amber-suppressing activity.

Ad2$^+$ND1 Host-Range Mutants. Previously, it was shown by cell-free translation of SV40-specific RNA that the host-range mutants of Ad2$^+$ND1 encoded specific polypeptide fragments rather than the M_r 30,000 protein of the parent (22). Two similar and perhaps identical mutants, 140 and 162, gave a M_r 19,000 fragment and mutant 71 gave a M_r 10,000 fragment. The cell-free suppression system was used to determine whether these fragments were generated by mutations to ochre or amber terminators. The autoradiograms shown in Figs. 3 and 4 display the products of cell-free protein synthesis in nuclease-treated reticulocyte lysates programmed by mRNA extracted from the cytoplasm of permissive (human) cells infected with adenovirus, Ad2$^+$ND1, or the host-range mutant viruses. The M_r 30,000 protein, specific for the ND1 hybrid, could be detected in the products of translation of the total cytoplasmic RNA (Fig. 3, lane ND1; Fig. 4, lane ND1), as could the M_r 19,000 fragment specific for host-range mutant 140 (Fig. 3, lane 140). These two polypeptides are the predominant products if the mRNAs were each enriched for SV40 sequences (Fig. 3, lanes Fractionated ND1 and 140). Note that the product from hybridized 140 RNA revealed a number of additional

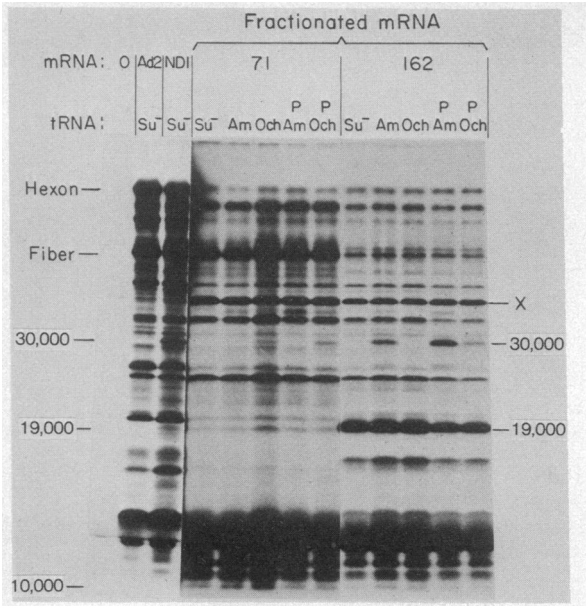

FIG. 4. NaDodSO$_4$/polyacrylamide gel electrophoresis of products of cell-free protein synthesis. The autoradiogram is a composite of different exposures: lanes 0, Ad2, and ND1, 2 days; lanes Fractionated 71, 10 days; lanes Fractionated 162, 4 days. See Fig. 3 for details.

proteins because of incomplete purification by the hybridization scheme, but these were not evident in the products from hybridized ND1 RNA because much less RNA was used for the translation.

Translation of the mutant RNA in the presence of crude tRNA from the nonsuppressing strain of yeast (Fig. 3, lane Fractionated 140, Su$^-$) yielded the M_r 19,000 product but no M_r 30,000 component (0.5% of M_r 19,000). However, crude tRNA from the amber-suppressing strain ("am") did restore partial synthesis of the M_r 30,000 protein (7% suppression), whereas crude tRNA from the ochre strain ("och") did not. Purified amber tRNA ("P am") greatly increased the percentage of M_r 30,000 protein in relation to M_r 19,000 protein (33% suppression), and the purified ochre tRNA ("P och") reaction showed a low level of M_r 30,000 protein synthesis (5%). The pattern of M_r 30,000 protein synthesis induced by these tRNAs paralleled the suppression observed when Qβ amber mutant RNA was used as template, as shown in Fig. 2. We conclude that the synthesis of M_r 30,000 protein directed by RNA extracted from the 140 mutant-infected cells is due to cell-free suppression of the mutant amber codon that otherwise causes termination to yield the M_r 19,000 fragment. Hence, the 140 mutant is an amber mutant.

Suppression of the host-range mutant 162, which also makes a M_r 19,000 fragment, is shown in Fig. 4 (lanes Fractionated 162). Again, the crude amber and the purified amber tRNAs restored synthesis of the M_r 30,000 protein, the crude *su*$^-$ and crude ochre tRNAs did not, and the purified ochre tRNA gave a low level of suppression. We conclude that host-range mutant 162 is also an amber mutant. Results with host-range mutant 71 are also shown in Fig. 4 (lanes Fractionated 71). The M_r 10,000 fragment produced by this mutant (22) could not be readily seen because the presence of large amounts of unlabeled globin in the reticulocyte lysate made it difficult to resolve small proteins on these gels and also because the level of its synthesis in the reticulocyte system, in contrast to the Schreier and Staehelin system, was low, even when the RNA had been enriched by hybridization to SV40 DNA. However, examination of the autoradiogram suggested that M_r 30,000 protein was synthesized in the presence of crude ochre and, especially, purified ochre tRNAs but not in the presence of *su*$^-$ or the two amber tRNAs. We conclude that the 71 mutation is a new ochre codon in such a position that it results in synthesis of a M_r 10,000 fragment.

We have done similar experiments in the Schreier and Staehelin cell-free system and have seen suppression of the 140 and 162 mutants with amber tRNA and suppression of 71 with ochre tRNA, although the presence of additional components near M_r 30,000 in the reaction products makes visualization more difficult (unpublished data).

DISCUSSION

Translation *in vitro* of mRNA from the Ad2$^+$ND1 host-range mutants 140 and 162 gives substantial amounts of wild-type-size ND1 M_r 30,000 protein in addition to the mutant M_r 19,000 fragment in the presence of amber tRNA from yeast, whereas the Ad2$^+$ND1 mutant 71 yields wild-type M_r 30,000 protein only in the presence of yeast ochre tRNA. The simplest interpretation of these results is that 140 and 162 are amber mutants and that 71 is an ochre mutant whose suppression *in vitro*, like that of Qβ bacteriophage nonsense codons, is mediated by tRNAs. One could argue that the mutations that result in synthesis of fragments of the ND1 M_r 30,000 protein are not nonsense mutations but are small deletions or frame-shift mutations that result in out-of-phase translation that ultimately terminates at an out-of-phase nonsense codon. In the case at hand, the suppressed products are the same size as the wild-type protein, within our experimental error, and one would have to argue that by chance the first terminator found in the new reading frame after suppression is placed such that a M_r 30,000 product is produced. This seems like a remote possibility, but it can ultimately be tested by further characterization of the protein. In any case, because the mRNA from mutant-infected cells can be translated beyond the mutant terminator site, the mRNA that is not being translated in the cell is not systematically degraded.

The existence of the Ad2$^+$ND1 nonsense mutants is entirely consistent with the fact that mammalian cells are known to use all three termination codons: UAA in human α- (35) and β- (36, 37) and rabbit α- (38) globins, UGA in rabbit β-globin (36, 39), and UAG in the Wayne frameshift variant of human β-globin (40) and in at least two adenovirus genes (unpublished data).

Protein synthesis, in various cell-free mammalian translation systems, obeys UAG and UAA termination signals both at the normal ends of cistrons and at internal mutant sites of bacteriophage RNAs (see *Results*) (5, 6, 41–43). Thus, no endogenous suppressor activity of amber or ochre codons has been detected in the mammalian cell extracts so far tested. The existence of the Ad2$^+$ND1 nonsense mutants allows us to examine cellular suppressor activity *in vivo*. Because we cannot detect synthesis of the ND1 M_r 30,000 protein in monkey cells infected with these mutants and because the mutants are "tight" mutants in the sense that the virus production in infected monkey cells is down to the low level seen in adenovirus-infected cells (22), it appears that the monkey cell lines we used do not have the ability to suppress either ochre or amber mutations at a level sufficient for us to detect. Similarly, we cannot detect synthesis of the M_r 30,000 ND1 protein in permissive HeLa cells infected with the host-range mutants (22) although, if the protein were synthesized at less than 10% of wild-type levels, it might be undetected.

It is hoped that identification of Ad2$^+$ND1 140, 162, and 71 as nonsense mutants will help to provide a first step in obtaining a functioning suppressor system in mammalian cells. In the case at hand, monkey cells provide the nonsuppressing host and in principle we could ask whether mutants of these monkey cells

can be found that are capable of allowing the growth of mutant virus. These then might contain nonsense suppressors. Unfortunately, there is no obvious way to select for such mutants, and screening large numbers of clones represents a formidable task. A different approach involves the introduction into monkey cells of a defective virus or other vector that carries a gene coding for a suppressor tRNA. The *Escherichia coli* amber suppressor, su^+ III, has been ligated to SV40 DNA and introduced into monkey cells (44). However, no suppressor tRNA product was detected although RNA complementary to suppressor gene sequences was produced. One might consider that a yeast suppressor gene would be more likely to function in mammalian cells because it might have the proper transcription and/or processing signals and because mammalian cells might have the enzymes needed for processing the transcribed tRNA. We do know that the yeast suppressors we have used can function in an otherwise completely mammalian system, at least *in vitro*, as long as there are enough copies of the tRNA to compete with the normal termination process.

Other genetic systems provide selective schemes that might yield suppressor cell lines. Capecchi *et al.* (45) have recently found a mutant of mouse cells that is deficient in hypoxanthine phosphoribosyltransferase and that can recover partial enzymatic activity when the cells are fused with erythrocytes preloaded with ochre suppressor tRNA. Revertants of these cells are potential suppressors. The Ad2$^+$ND1 amber and ochre mutant viruses can be used to screen these revertant lines as well as revertants of other hypoxanthine phosphoribosyltransferase mutants (46) and revertants of mouse myeloma cell heavy chain immunoglobin mutants (47) and human cell mutants in which herpes virus thymidine kinase mutants will grow (48). Because none of these schemes involves the use of monkey cells, it is not possible to detect suppressor activity directly by asking whether the revertant cell lines are capable of supporting the growth of the host-range mutants described here. In principle, however, one can look for the synthesis of the suppressed ND1 M_r 30,000 protein itself in the infected revertant cells.

Whether or not a mammalian cell can survive with a suppressor gene in it is still completely unclear. The fact that yeast cells can carry suppressors and remain viable and healthy is encouraging.

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